

A bulky modification on the rim of azurin's β -barrel results in folded protein

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Abstract *Pseudomonas aeruginosa* azurin is a blue-copper protein with a β -barrel fold. Here we report that, at conditions where thermal unfolding of apo-azurin is reversible, the reaction occurs in a single step with a transition midpoint (T_m) of 69°C (pH 7). The active-site mutation His117Gly creates a cavity in the β -barrel near the surface but does not perturb the overall fold (T_m of 64°C, pH 7). Oxidation of the active-site cysteine (Cysteine-112) in wild-type azurin, which occurs readily at higher temperatures, results in a modified protein that cannot adopt a native-like structure. In sharp contrast, Cysteine-112 oxidation in His117Gly azurin yields a modified apo-azurin that appears folded and displays cooperative, reversible unfolding ($T_m \sim 55^\circ\text{C}$, pH 7). We conclude that azurin's β -barrel is a rigid structural element that constrains the structure of its surface; a bulky modification can only be accommodated if complementary space is provided.

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Key words: Protein folding; Azurin; Thermal denaturation; Differential scanning calorimetry; Cysteine oxidation; β -barrel stability

1. Introduction

Whenever Nature uses copper to promote biological electron transfer, the metal appears to be incorporated in a cupredoxin fold (e.g. azurin, plastocyanin, amicyanin, nitrite reductase and laccase). This fold is usually made up of 6–8 β -strands arranged in a Greek key-folding motif. The copper-binding site is strictly conserved and always located eccentrically at a distance of 5–7 Å from the outer surface. The metal is anchored in the protein structure by strong bonds to three ligands in a trigonal plane or trigonal pyramidal geometry. A weaker fourth ligand, and sometimes a fifth (as is the case for azurin), occupies the axial position(s). In *Pseudomonas aeruginosa* azurin, the copper is coordinated in one plane by the side-chains of Cysteine-112, Histidine-117 and Histidine-46 [1]. At the axial positions, the copper interacts with the sulfur of Methionine-121 and the carbonyl oxygen of Glycine-45. Cysteine-112 and Methionine-121 are at the beginning and

end, respectively, of the ligand-binding loop, which also contains Histidine-117. Thus, the carbonyl group of Glycine-45 and the side-chains of Histidine-46, Methionine-121 and Cysteine-112 are firmly buried in the β -barrel. In contrast, the imidazole ring of Histidine-117 protrudes through the protein's surface.

The crystal structures of copper-, zinc- and apo-azurin have all been determined with high resolution [2–4]. There is no significant structural difference between the proteins, confirming that azurin adopts a native β -barrel structure with and without the metal ion. Many active-site mutations have been implemented in azurin in order to study the effects on the blue-copper site, and its electron-transfer properties upon ligand substitutions. One of these azurin variants is His117Gly (histidine-to-glycine mutation at position 117), which creates a channel leading from the bulk solvent directly to the hydrophobic core. Extended X-ray absorption fine structure and NMR studies showed that the mutation does not perturb the structure and dynamics of the β -barrel [5]. However, upon crystallization of His117Gly azurin, Cysteine-112 was fully oxidized to sulfonic acid. The structure of this modified form was found to deviate largely from wild-type azurin in the loops connecting the β -strands of the β -barrel. Still, the β -barrel remained intact [6].

Thermal denaturation studies of azurin (various metallo- as well as the apo-form) have been hampered by the irreversibility of the reactions as monitored by DCS [7]. In a recent report, the nature of the thermally induced unfolding exhibited by azurin was investigated [8]. It was concluded that under aerobic conditions the major source of irreversibility of unfolding was oxidation of the thiolate of Cysteine-112 to form inter-molecular disulfide bond in the thermally denatured state. By elimination of molecular oxygen, or removal of Cysteine-112 by mutagenesis, reversible transitions for Cu(I), Zn(II)-, and apo-variants of the cysteine mutants of azurin were observed. Wild-type apo-azurin was however not included in this study. This is important, since earlier findings on apo-azurin [7] suggested that its thermal unfolding reaction consists of two well-separated irreversible transitions (at about 62 and 82°C, pH 7). Disappearance of the secondary structure (far-UV circular dichroism (CD)) was observed at temperatures corresponding to the second differential scanning calorimetry (DSC) transition. The origin of the low-temperature transition was not clarified but it was suggested that this endothermic peak could have occurred due to a 'flap' of the small α -helix and associated with its exposure of a hydrophobic surface on one of the β -strands [7].

Here we report that at conditions where thermal unfolding

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Abbreviations: GuHCl, guanidine hydrochloride; DSC, differential scanning calorimetry; CD, circular dichroism; T_m , midpoint of thermal transition

of apo-azurin is reversible the reaction occurs in a single step, as monitored by DSC and far-UV CD. In addition, we have created and studied thermodynamic stability of the His117Gly azurin variant in which Cysteine-112 is oxidized to sulfonic acid. In contrast to wild-type azurin with this modification, the modified His117Gly variant is folded and displays cooperative, reversible unfolding. Apparently, azurin's β -barrel core is a rigid structural element that constrains the structure of its surface; a bulky modification can only be accommodated if complementary space is provided.

2. Materials and methods

2.1. Protein expression and purification

The pUC18-derived plasmids carrying wild-type azurin and the point mutation His117Gly (replacement of Histidine-117 by glycine) were a generous gift from Dr. G. Karlsson (University of Gothenburg, Sweden).

Wild-type and His117Gly azurin variants were expressed in *Escherichia coli* strain RV308 as previously described [9–11]. In brief, the cells were grown in Luria–Bertani medium supplemented with 100 μ M ampicillin. Expression of azurin was induced by addition of isopropyl- β -D-thiogalactoside (0.5 mM) when the OD_{600nm} reached 0.6. The cell paste was re-suspended in a sucrose buffer (20% sucrose, 0.3 M Tris–HCl buffer, pH 8.1) and subjected to the osmotic shock (0.5 mM MgCl₂), resulting in a periplasmic protein slurry. Contaminating proteins were acid-precipitated from the periplasmic preparation by decreasing the pH to 4.1 (50 mM ammonium acetate buffer), yielding an azurin-containing supernatant that was loaded onto a SP Sepharose HP (Sigma) ion exchange column. Azurin was eluted by a pH gradient from 4.5 to 9.0 (50 mM NH₃–HAc buffer). Following concentration via PEG dialysis, gel filtration on Superdex 75 (Pharmacia) column yielded 95–97% pure apo-azurin. To obtain the modified His117Gly azurin (with Cysteine-112 oxidized), CuSO₄ was added to the crude protein sample before pH precipitation. The purity of the azurin variants was confirmed by gel electrophoresis on 4–12% Bis–Tris gel (Invitrogen). Protein concentration was determined spectroscopically by measuring optical absorption at 280 nm ($\epsilon_{280} = 9000 \text{ M}^{-1} \text{ cm}^{-1}$). For wild-type apo-azurin and non-modified His117Gly azurin, the purity with respect to metal contamination was tested by a Cu(II)-reconstitution assay, in which upon addition of CuSO₄ to the protein the intensity of the charge-transfer absorption in the visible region was compared to the aromatic absorption at 280 nm [9,12]. For wild-type azurin, the spectral ratio A_{630}/A_{280} is greater than 0.52; for non-modified His117Gly azurin, $A_{420}/A_{280} > 0.22$.

Modification of the active-site cysteine, Cysteine-112, in His117Gly azurin results in a protein incapable of copper binding. In order to eliminate the possibility of zinc contamination in this protein sample, the protein (50 μ M) was incubated for 60 min in 6 M guanidine hydrochloride (GuHCl) with addition of a metal-chelating agent (1 mM EDTA), then allowed to refold upon dialyzing against 5 mM PO₄ buffer (3 h). The renatured protein (complete restoration of the native secondary structure as probed by far-UV CD) did not bind externally added copper. The absence of a free thiol in modified His117Gly mutant was verified by Ellman's assay [13]. Moreover, to confirm that Cysteine-112 was fully oxidized to sulfonic acid (SO₃[−]) in the modified His117Gly azurin, we performed MALDI (matrix-assisted laser desorption/ionization) mass spectroscopic analysis. The molecular weight of the modified protein species (13,904 Da) is 44 mass units above the calculated molecular weight of non-modified apo His117Gly azurin, which corresponds (within experimental error) to three additional oxygen atoms (48 mass units).

2.2. Thermal unfolding monitored by DSC

Prior to thermal experiments monitored by DSC all samples were degassed using a ThermoVac accessory unit (MicroCal). The sample and reference cells were loaded according to the manufacturer's specified procedure. Prescan equilibration time was 30 min. To obtain a baseline both cells were filled with buffer and scanned from 25 to 108°C at scan rate of 40–90°C/h. At least three buffer-versus-buffer scans were taken to obtain a reproducible baseline. Every protein solution was scanned three times in order to determine the degree

of the reversibility of unfolding. The DSC scans were carried out under a pressure of 30 psi. To provide for highest sensitivity, passive (no cell–cell compensation) mode was used for the thermal equilibration of the reference and the sample cells. The average level of noise was $\pm 1 \mu\text{cal/min}$ (0.07 μwatts). The DSC scans were analyzed using

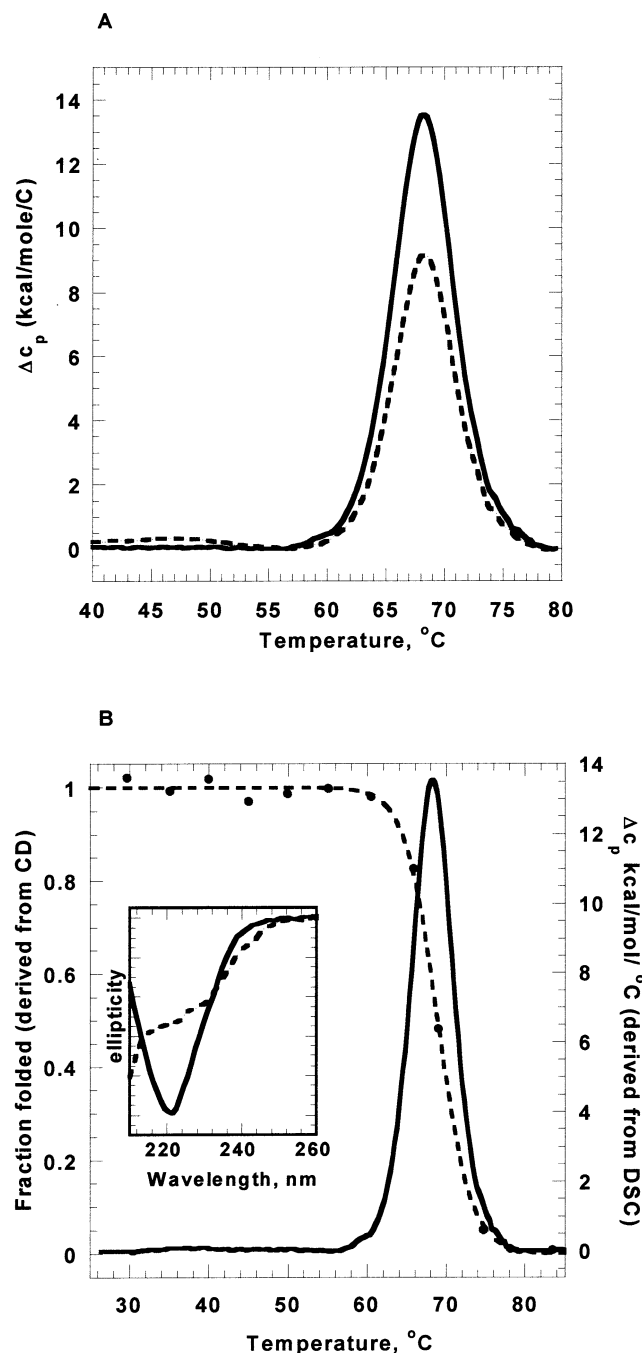


Fig. 1. A: Two sequential DSC thermograms, first scan (solid line) and second scan (dashed line), of wild-type apo-azurin (36 μ M apo-azurin; 5 mM phosphate buffer, pH 6). The sample was scanned from 20 to 85°C at a scan rate of 60°C/h. The appearance of the thermal transition at the same temperature upon rescans indicates that the unfolding is reversible. B: Thermally induced unfolding transition of wild-type apo-azurin (dotted line) monitored by far-UV CD (220 nm; 20 μ M apo-azurin; 5 mM phosphate buffer, pH 6) overlaid with the DSC thermogram (solid line) from Fig. 1A. Inset, CD spectra of folded (25°C; solid line) and unfolded (85°C; dotted line) wild-type apo-azurin.

Origin software (MicroCal). First, the buffer-versus-buffer reference scan (at the corresponding scan rate) was subtracted from the DSC sample curves. Next, the data was normalized to the concentration of the protein. A two-state model was used for data analysis. From the fit, the midpoint of the thermal transition (T_m) and the van't Hoff enthalpy change (ΔH_{vH}) were obtained. Protein concentration of 25–40 μM was used in the DSC experiments. No concentration dependence was observed in the studied range. Phosphate buffer pH 6.0 (5 mM) was used in all DSC samples. Percent reversibility was estimated from the ratio of areas under the thermal transitions of first and second scans.

2.3. Thermal unfolding monitored by far-UV CD

Thermal unfolding monitored by far-UV CD was performed in circular cell with 1-mm path using an Olis spectrophotometer equipped with a programmable temperature-controlled water bath and an external temperature probe. The samples were pre-equilibrated at 20°C and then heated up to 95°C (if not specified otherwise). At each temperature, a spectrum from 260 to 210 nm was recorded. Also, the CD experiments were performed using phosphate buffer pH 6.0 (5 mM). Protein concentration was 20–35 μM . Copper-reconstitution experiments showed that cold-renatured wild-type and His117Gly apo-azurins are able to bind copper like the native (non-heated) forms. Percent reversibility is estimated from the return of CD signal upon cooling to 20°C.

2.4. GuHCl-induced unfolding at 20°C

GuHCl-induced unfolding reactions of azurin variants (10–20 μM protein concentration; 5 mM phosphate buffer, pH 7) were monitored by far-UV CD and fluorescence as a function of GuHCl concentration. Tryptophan fluorescence was measured using a Varian eclipse spectrometer with excitation at 285 nm (5 nm band-pass) and emission at 308 nm (5 nm band-pass) at 20°C. CD was measured from 200 to 260 nm using rectangular cell with 1-mm path. The unfolding transitions were analyzed using a two-state model, $f_F + f_U = 1$, where f_F and f_U represents the fraction of total protein in the folded and unfolded conformations, respectively. Non-linear least-squares fits to the equilibrium data were generated using KaleidaGraph to fit the following expression:

$$Y_X = (Y_U - Y_F(\exp((\Delta G_U(0 \text{ M GuHCl}) - m[\text{GuHCl}])/RT))) / (1 + (\exp((\Delta G_U(0 \text{ M GuHCl}) - m[\text{GuHCl}])/RT)))$$

where Y_X , Y_U and Y_F are the observed spectroscopic signal at X M GuHCl, unfolded-protein baseline and the folded-protein baseline, respectively. From the fits, the unfolding free energy in the absence of denaturant, $\Delta G_U(0 \text{ M GuHCl})$ and m values were determined. The parameter m is a measure of cooperativity of the unfolding process and it can be correlated with the difference in solvent exposure of the protein's hydrophobic groups in the native and unfolded states [14,15]. The transition midpoints were calculated as $\Delta G_U(0 \text{ M denaturant})/m$, or by direct inspection of the transitions.

3. Results and discussion

3.1. Reversible thermal unfolding of wild-type and His117Gly apo-azurins

Thermal unfolding of *P. aeruginosa* azurin (apo, as well as various metal-substituted forms including Cu(II)) has previously been reported to be irreversible as monitored by DSC

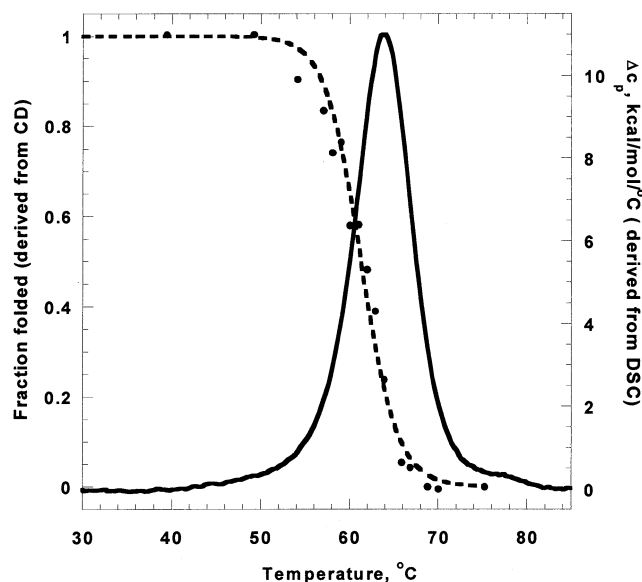


Fig. 2. Thermally induced unfolding of non-modified His117Gly apo-azurin (5 mM phosphate buffer, pH 6) as monitored by DSC (solid curve) and far-UV CD at 220 nm (dashed curve is the two-state fit to the experimental data points, see [Materials and methods](#)). The concentration of apo His117Gly azurin was 22 μM and 37 μM in CD and DSC experiments, respectively.

[7]. Part of the irreversibility upon thermal unfolding was attributed to Cysteine-112 oxidation at the higher temperatures due to transition-metal catalyzed oxidation with dissolved oxygen [8].

Upon careful investigating of the pH- and buffer-dependence for the reversibility of thermal unfolding of wild-type apo-azurin, we concluded that a significant contribution to the irreversibility must come from deamidation and isoaspartate formation reactions, the rates of which are known to be temperature- and pH-dependent [16]. *P. aeruginosa* azurin contains seven asparagine and 11 aspartic acid residues. They are scattered along azurin's primary sequence and occur within the β -barrel as well as in the loop regions of the protein. The covalent modifications of all or some of these residues at high temperatures can result in failure of azurin to adopt its native structure upon subsequent cooling. We found that in phosphate buffer, pH ≥ 6 , wild-type apo-azurin unfolds reversibly under *aerobic* conditions. In contrast to previously reported irreversible data [7], wild-type apo-azurin unfolds reversibly in a single transition with a T_m of $69 \pm 0.1^\circ\text{C}$ corresponding to an enthalpy change, $\Delta H(T_m)$, of $543 \pm 20 \text{ kJ/mol}$ (Fig. 1A, Table 1). As expected, these parameters are lower than are those for metal-substituted forms [7,8]. We also probed the thermal unfolding process of apo-azurin by

Table 1
Thermal unfolding of wild-type and His117Gly apo-azurins as probed by DSC and far-UV CD at reversible conditions

Azurin:	DSC			CD	
	T_m ($^\circ\text{C}$)	$\Delta H(T_m)$	% rev.	T_m ($^\circ\text{C}$)	% rev.
Wild-type	68.9 ± 0.2	$543 \pm 4 \text{ kJ/mol}$	70	68 ± 1	70
His117Gly	63.7 ± 0.2	$412 \pm 5 \text{ kJ/mol}$	60	62 ± 1	65
Mod. wild-type	Not folded	–	–	Not folded	–
Mod. His117Gly	55.5 ± 1	$196 \pm 15 \text{ kJ/mol}$	75	52 ± 1	75

Proteins with non-oxidized Cysteine-112 are compared to the cysteine-modified forms (modified wild-type and modified His117Gly azurins).

far-UV CD. In good agreement with DSC data, the secondary structure of azurin disappears in a single transition that parallels that monitored by DSC (Fig. 1B). Although the CD signal suggests that unfolded apo-azurin has some residual structure, the CD spectrum does not change further upon heating to 100°C. This is in good agreement with the absence of any DSC transitions at temperatures higher than 69°C. Thus, thermal unfolding of apo-azurin, under reversible conditions, is not a multi-step reaction with an intermediate as previously suggested [7].

The active-site mutant His117Gly azurin exhibits the same behavior as wild-type apo-azurin: the thermal unfolding is a reversible two-state process with overlapping CD and DSC transitions (Fig. 2, Table 1). Removal of Histidine-117 creates a channel from the surface of the protein to the active site that is occupied by water molecules. We have previously shown that this modification results in a protein with wild-type properties but that is less stable towards GuHCl-induced unfolding ($\Delta G(\text{H}_2\text{O})$ decreased by $\sim 30\%$ as compared to wild-type [17]; Table 2). In good agreement, we find that the thermal midpoint is decreased by ~ 5 degrees and $\Delta H(T_m)$ is decreased by $\sim 20\%$ as compared to wild-type apo-azurin data (Table 1). The destabilizing effect of the mutation can be explained by the facilitated access of bulk solvent to the protein's hydrophobic interior. For comparison, a cavity-creating substitution of Cysteine-112 for alanine resulted in a mutant azurin as stable as His117Gly azurin: T_m for Cys112Ala azurin is reported to be $\sim 64^\circ\text{C}$ at pH 6.2 [8]. Replacement of Cysteine-112 by a serine, a residue with a side chain of approximately the same size as that of cysteine, does not introduce a cavity and, in accordance, the corresponding mutant, Cys112Ser azurin, did not show any decrease in thermal stability (T_m of $68.9 \pm 0.1^\circ\text{C}$; pH 6.3) [8] as compared to wild-type apo-azurin (T_m of $69 \pm 0.2^\circ\text{C}$; pH 6.0).

3.2. Cysteine-112 oxidation in wild-type and His117Gly variants

It has been reported that upon aerobic thermal denaturation of wild-type Cu(I)- and Cu(II)-azurin 25% of the protein was found to have Cysteine-112 oxidized to sulfonic acid [8]. The resulting modified azurin could not refold upon cooling. Thus, azurin cannot accommodate a bulky sulfonic acid on the rim of the β -barrel in its wild-type form. We also confirmed this conclusion. DSC of wild-type apo-azurin in the presence of sub-stoichiometric amounts of added Cu(II) resulted in a first thermal scan which shows, as expected, thermal transitions for apo- and Cu(II)-forms of azurin (68.1°C and 86.5°C ; phosphate buffer, pH 6). The subsequent thermal scan shows a peak only positioned at the expected T_m for Cu(I) azurin (T_m 85.4°C , [8]). Finally, the third scan shows no thermal transitions at all. This experiment implies that the oxidation reaction between Cu^{2+} and the sulfur of

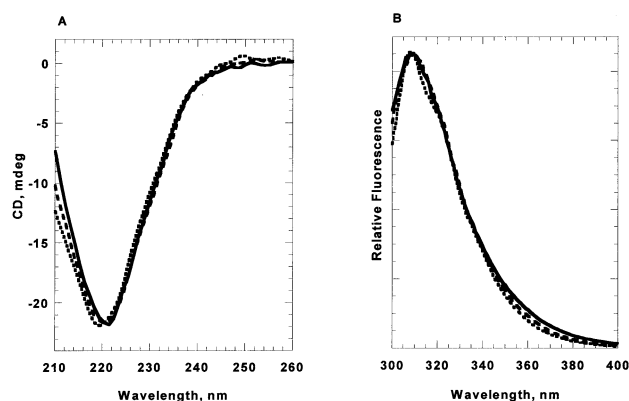


Fig. 3. Far-UV CD spectra (A) and normalized fluorescence emission spectra through excitation at 295 nm (B) of native forms of modified His117Gly (dotted line), non-modified His117Gly (dashed line) and wild-type (solid line) apo-azurins (5 mM phosphate buffer, pH 6, 20°C).

Cysteine-112 produces Cu(I) and sulfonate-modified Cysteine-112 ($-\text{SO}_3\text{H}$) in the thermally unfolded state of wild-type azurin. Upon cooling, the fraction of apo-azurin that has not been involved in the oxidation process refolds and binds Cu^{1+} , producing Cu(I)-loaded azurin. On the second round of heating Cu(I)-azurin gets modified at Cysteine-112 in an oxidation reaction now mediated by Cu(I). In the third scan all protein molecules have become modified at Cysteine-112 so that there is no folded protein in the sample and, therefore, no energetic transition is observed upon heating. In accord with cysteine oxidation to sulfonic acid, and not dimerization through inter-molecular disulfide formation, gel electrophoresis of heated (in presence of fraction Cu(II)) wild-type azurin samples show monomers, and no dimeric species (data not shown).

In sharp contrast to the wild-type azurin experiment described above, re-heating of His117Gly azurin samples containing sub-stoichiometric amounts of Cu^{2+} results in a broad transition that overlaps with that observed for purified Cysteine-112 oxidized His117Gly azurin (see below).

We discovered Cysteine-112 oxidized His117Gly azurin when CuSO_4 was added in the early stages of protein purification (prior to pH precipitation). Various biochemical and analytical tests prove that Cysteine-112 is oxidized to sulfonic acid, but the rest of the polypeptide is intact in this modified form (see Materials and methods). Cysteine-112-modified His117Gly azurin exhibits wild-type secondary structure and tryptophan emission in buffer solution (Fig. 3A, B). The unique, structured tryptophan-48 emission of apo-azurin is a result of its deep burial within azurin's hydrophobic core. Since the modified mutant exhibits the same emission as wild-type apo-azurin, we conclude that the core must be intact

Table 2
GuHCl-induced unfolding of wild-type, non-modified His117Gly and modified His117Gly apo-azurins (pH 7, 20°C)

Azurin:	$\Delta G(\text{H}_2\text{O})$	$[\text{GuHCl}]_{1/2}$	m
Wild-type	29.1 ± 1.8 kJ/mol	1.7 ± 0.1 M	17.5 ± 1.1 kJ/mol, M
His117Gly	19.2 ± 1.0 kJ/mol	1.2 ± 0.1 M	16.0 ± 0.5 kJ/mol, M
Mod. His117Gly	12.4 ± 0.9 kJ/mol	1.1 ± 0.2 M	11.0 ± 0.8 kJ/mol, M

Parameters were derived from two-state fits, see Materials and methods. Data for wild-type and His117Gly azurins were taken from elsewhere [11,17]. In all three cases, far-UV CD and fluorescence-monitored transitions give identical results.

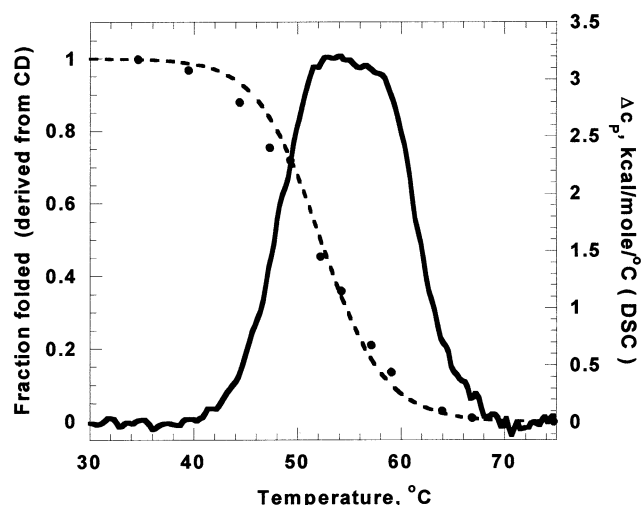


Fig. 4. Thermal unfolding of modified His117Gly apo-azurin (5 mM phosphate buffer, pH 6) as monitored by DSC (solid curve) and far-UV CD at 220 nm (dashed curve represents the fit to the experimental data points using a two-state model, see [Materials and methods](#)). The protein concentration was 20 μ M and 37 μ M in the CD and DSC experiments, respectively.

in the modified mutant. This Cysteine-112 oxidized azurin form has never been observed in solution before.

3.3. Characterization of modified His117Gly azurin

The thermal unfolding of Cysteine-112-oxidized His117Gly azurin is shown in Fig. 4. The single unfolding transition is rather broad compared to wild-type and non-modified His117Gly azurins. As is the case for wild-type and His117Gly apo-azurins, the modified His117Gly mutant exhibits overlapping far-UV CD and DSC-monitored thermal transitions. The protein stability is decreased by the introduced bulky modification, as is evident from a lower T_m and a decrease in $\Delta H(T_m)$ (Table 1). Still, the unfolding transition is cooperative, which is indicative of a well-behaved folded protein.

We also performed GuHCl-induced unfolding of the modified His117Gly azurin at 20°C. Far-UV CD and fluorescence-monitored unfolding data overlap, resulting in a transition midpoint lower than the ones for wild-type and non-modified His117Gly apo-azurins (Table 2). Both wild-type and His117Gly azurins display similar m -values, suggesting that the same amount of hydrophobic surface area is exposed upon unfolding and, therefore, the folded states are packed to the same degree [18]. In the case of the modified His117Gly form, the m -value is lowered by 30% (Table 2), suggesting different packing around the modification and/or a less well-packed core.

3.4. Correlation to crystallographic data on modified His117Gly azurin

The reported crystal structure of modified apo His117Gly azurin in which Cysteine-112 had become fully oxidized during the crystallization procedure [6] provides an explanation to the experimental solution data presented here. The bulky modification of Cysteine-112 is found to impose significant rearrangements in the 'northern' end (copper-binding region) of azurin. Cysteine-112 is on the rim of the β -barrel and one would expect that a bulky modification there would cause

large perturbations in the β -barrel. However, major structural changes upon Cysteine-112 oxidation only occur in the conformation of the loops connecting the β -strands in azurin, whereas the β -barrel remains essentially intact. In order to accommodate the sulfonic moiety, the copper-binding loop (residues 113–121) has assumed a different conformation that provides the necessary space and makes possible a hydrogen-bonding network (distinct from the one in wild-type apo-azurin) which decreases the partial negative charges on the oxygen atoms of the sulfonic acid. The replacement of Histidine-117 with a glycine adds plasticity to this peptide loop and allows the necessary structural reorganizations to take place. The fact that wild-type azurin fails to fold upon Cysteine-112 oxidation suggests that the loops connecting the β -strands are packed tightly and, despite their lack of secondary structure, have restricted conformational freedom.

3.5. Summary

Our investigation of the thermodynamic stability of His117Gly azurin in which the active-site cysteine is oxidized to sulfonic acid shows that azurin's β -barrel is robust and supports formation of a stable protein despite the large modification. However, our study also reveals that the loops on the rim of azurin's β -barrel are tightly packed and must be restricted in motion. A bulky modification, such as sulfur oxidation, can only be accommodated within the protein scaffold when complementary space is provided (such as upon Histidine-117 elimination). The use of a β -barrel as a rigid support is found in many electron-transfer proteins (especially cupredoxins) [19]. It appears as if the protein structure does not enforce a 'rack' onto the copper ion in the active site but instead provides a robust anchor for the active-site residues and the connecting loops.

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